Original paper

Treponema pallidum-specific immune responses and autoimmunity in patients who remain serofast after treatment of syphilis

Maciej Pastuszczak[1], Agnieszka Kotnis-Gąska[2], Bernadetta Jakubowicz[3], Anna Wojas-Pelc[1]

[1]Department of Dermatology, Jagiellonian University Medical College, Krakow, Poland
[2]Department of Laboratory Diagnostics, John Paul 2nd Hospital, Krakow, Poland
[3]Department of Microbiology, University Hospital, Krakow, Poland

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Abstract

Introduction: Approximately 15% of appropriately treated patients with early syphilis remain serofast. The pathogenesis and clinical significance of this phenomenon are unclear.
Aim: To determine the significance of *Treponema pallidum*-specific immune responses and autoimmunity in the treatment outcome of syphilis (serofast or proper serological response).
Material and methods: Forty-eight patients with secondary and early latent syphilis (ELS) were enrolled in this study. Reactivity of IgM/IgG antibodies to the treponemal antigens TpN47, TpN17, TpN15 and TmpA was evaluated before and 12 months after intramuscular penicillin therapy for syphilis. Additionally, the presence of antinuclear antibodies (ANA) was determined 12 months after treatment.
Results: After 1 year, patients were stratified into two groups based on their serological response: (1) serofast (n = 10) and (2) serologically-cured (n = 38) patients. The serological cure rate was 79.2% at 12 months after treatment. Weak pre- and post-treatment antibody reactivity to TpN47 antigen was found to be significantly associated with a higher risk of the serofast state (OR = 64; 95% CI: 5.01–817; $p < 0.005$). Patients who remained serofast had a significantly higher ANA prevalence and mean titer when compared to those with proper serological responses (100% vs. 5.3%, respectively, $p < 0.005$; 1 : 640 vs. 1 : 160, respectively, $p < 0.005$).
Conclusions: We demonstrate that baseline antigen-specific immune response to Treponema pallidum may be an important predictor of the treatment outcome. Further studies are warranted to identify the role of autoimmunity in the pathomechanism of the serofast state.

Key words: syphilis, serofast, autoimmunity, immune response.

Introduction

Parenteral penicillin is the therapy of choice at every stage of syphilis, but due to inability to culture *Treponema pallidum in vitro*, monitoring of treatment efficacy relies almost entirely on the measurement of the immune response (i.e. serological assays) rather than direct *T. pallidum* tests. Appropriate serologic response in syphilis has been defined by Centers for Disease Control and Prevention (CDC) as a four-fold or greater decline in the titer of non-treponemal assays when performed 6 months after beginning of the treatment [1]. However, approximately 15% of patients with early syphilis do not follow the classical patterns of serological response to therapy, exhibiting less than a four-fold decline in non-treponemal titers without evidence of treatment failure or reinfection [2].

Until now, only a limited number of studies have been conducted to investigate serological responses after syphilis treatment. In these studies, several factors have been implicated as predictors of improper serological response to treatment, such as older age of patients, lower baseline non-treponemal antibody titers and lack of appearance of Jarisch-Herxheimer reaction [3–5]. These studies, however, did not establish the clinical and biological explanation for the serofast state. The absence of novel methods to con-

Address for correspondence: Maciej Pastuszczak MD, PhD, Department of Dermatology, Jagiellonian University Medical College, 8 Skawińska St, 31-066 Krakow, Poland, phone: +48 602 228 796, e-mail: mpastuszczak@wp.pl
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firm eradication of *T. pallidum* leads to uncertainty regarding whether 'serofast state syphilis' is a signature of a persistent infection or simply a residual immune response in the absence of the viable pathogen. Thus, a majority of serofast patients undergo additional treatment despite lack of any evidence to support this practice.

Aim

To better understand and investigate the serofast condition, we analyzed an antigen-specific immune response that occurs during the infection and after the therapy. Since non-treponemal antibodies were identified as autoreactive particles [6], the second aim of the current study was to demonstrate a presumptive link between infection with *T. pallidum* and autoimmunity.

Material and methods

Patients and study design

Secondary and early latent syphilis (ELS) patients (n = 50) in their first episode of disease were enrolled at the Department of Dermatology of the Jagiellonian University Medical College in Krakow, Poland between 2015 and 2017. All patients included in the study were positive both for non-treponemal and treponemal tests at enrollment. Syphilis clinical staging was determined by a board-certified dermatologist using the Centers for Disease Control and Prevention (CDC) criteria [1]. After blood sampling for laboratory tests, including treponemal-specific tests (INNO-LIA Syphilis Score Assay), patients were administered intramuscular benzathine penicillin (2.4 million units). All study subjects were tested for concurrent HIV infection. Antiretroviral therapy was introduced in all patients with confirmed HIV co-infection. All cases of HIV infection were detected at enrollment, as none of the study patients were aware of their HIV status. After completion of the treatment, patients returned every 3 months for follow-up assessment (including non-treponemal testing; rapid plasma regain assay – RPR). Six months after completing the treatment, serological responses were assessed. The data indicated that 14 of 50 individuals did not achieve proper serological response, defined as at least a four-fold decline in RPR titer when compared to the pre-treatment values. In all of the subjects, cerebrospinal fluid (CSF) examination was performed. Diagnosis of neurosyphilis was determined according to CDC guidelines (i.e. reactive CSF VDRL or CSF pleocytosis of ≥ 5/µl and CSF protein concentration ≥ 45 mg/dl). In 2 cases, asymptomatic neurosyphilis was confirmed and these patients were excluded from further analysis. Re-treatment with intramuscular benzathine penicillin (2.4 million units) was administered in the remaining 12 patients. Twelve months after treatment, two of these twelve individuals achieved proper serological response. All patients studied were classified as (1) the serofast state group (n = 10) defined as the failure of the RPR titer to show a four-fold decline between the baseline and the 12-month visit and (2) the serologically-cured group (n = 38) defined as at least a four-fold decline in the RPR titer in comparison to the pre-treatment RPR results. Twelve months after treatment, in all the remaining individuals, serum samples for INNO-LIA Syphilis Score testing and antinuclear antibodies (ANAs) were collected. The study was approved by the Jagiellonian University Bioethics Committee (approval number KBET/164/B) and written informed consent was obtained from all participants.

Laboratory measurements

The RPR assay (Becton Dickinson & Company, Sparks, MD, USA) and the INNO-LIA Syphilis Score Assay (Fujirebio Europe N.V., Gent, Belgium) were performed according to the manufacturer's instructions. The INNO-LIA Syphilis Score Assay detects IgM/IgG antibodies specific for treponemal antigens TpN47 (47 kDa), TpN17 (17 kDa), TpN15 (15 kDa) and TmpA (42 kDa) (pool of recombinant antigens). The interpretation of the results was performed according to the instructions provided by the manufacturer. Briefly, a rating from 0 to 3+ was given to the antigen lines (bands) on the developed strips by comparing their intensities with those of the control lines. Weak, moderate and strong intensity corresponded with the 1+, 2+ and 3+ ratings, respectively.

ANA was detected by indirect immunofluorescence on HEp-2 cells. The assessment of autoantibody titers and five main patterns (granular, homogenous, nucleolar, centromere and other patterns) were carried out with a semi-automated system (AKLIDES®; Medipan GmbH, Dahlewitz, Germany) and confirmed by visual observation. Sera assessed as positive (titer ≥ 1 : 160) were further analyzed by specific second-step autoantibody assays according to the staining pattern. The selection of the confirmatory assays was carried out according to test algorithms of routine diagnostics.

Statistical analysis

Statistical analysis was carried out using Statistica 7.1 PL software (TIBCO Software Inc. Palo Alto, CA). Unless stated otherwise, data were expressed as median and minimum-maximum values. Continuous variables were compared with the Mann-Whitney U test. The χ^2 test or the Fisher's exact test was used for the dichotomous variables. Correlations were assessed using linear regression analysis (Pearson). A p-value < 0.05 was considered statistically significant.

Results

Patient characteristics

We enrolled 50 patients with the first episode of early syphilis, who were staged as secondary syphilis (50%) or

ELS (50%). Newly diagnosed HIV co-infection was found in ten individuals (20%). Six months after completing syphilis treatment, 14 (28%) patients did not achieve an at least four-fold decline in RPR titer. In 2 of them (14.3%), asymptomatic neurosyphilis was confirmed, what may explain the improper serological response. These 2 patients were excluded from further follow-up. All 12 remaining patients were retreated with intramuscular benzathine penicillin (2.4 million units). Six months after retreatment, only 2 (16.7%) individuals demonstrated a decrease in the RPR titer and met the criteria of proper serological response (i.e. at least four-fold decline in RPR titer in comparison to pre-treatment RPR results).

Twelve months after completing the syphilis treatment, the patients were stratified into: (1) the serofast state group (n = 10), and (2) the serologically-cured group (n = 38). Patients from the serofast state group had a lower baseline RPR titer when compared to individuals from the serologically-cured group. The groups did not differ with respect to sex, age, clinical stage of syphilis and frequency of concomitant HIV infection (Table 1).

Serum antibody reactivity to protein antigens of *T. pallidum* before and after syphilis treatment

All analyzed antigens (i.e. TpN47, TpN17, TpN15 and TmpA) were present in all syphilitic patients before treatment. In most individuals, pre-treatment intensity of the reaction for TpN17, TpN15 and TmpA was assessed as moderate or strong (i.e. 2+ or 3+). In contrast, diverse intensities of bands were observed for TpN47. Among all studied patients, a comparable number of patients with weak, moderate and strong reactivity for TpN47 were found (Figure 1 A).

In all patients, antibodies to the full complement of treponemal antigens were still demonstrable 1 year after treatment. The antibody reactivity to the antigens had not changed in comparison to the pre-treatment results (Figure 1 B).

Table 1. Characteristics of patients from the serofast state and serologically-cured groups

Parameter	Serofast state group (n = 10)	Serologically-cured group (n = 38)	P-value
Sex, males, n (%)	8 (80)	37 (97.4)	NS
Age [years]	32 (20–41)	33 (19–39)	NS
HIV co-infection, n (%)	2 (20)	7 (18.4)	NS
Clinical stage of syphilis, n (%):			NS
Secondary	5 (50)	22 (57.9)	
Early latent	5 (50)	14 (42.1)	
Baseline RPR titer	16 (4–512)	64 (8–256)	NS

HIV – human immunodeficiency virus, RPR – rapid plasma regain, NS – not significant. The values are presented as medians (min.-max. range) if not otherwise stated.

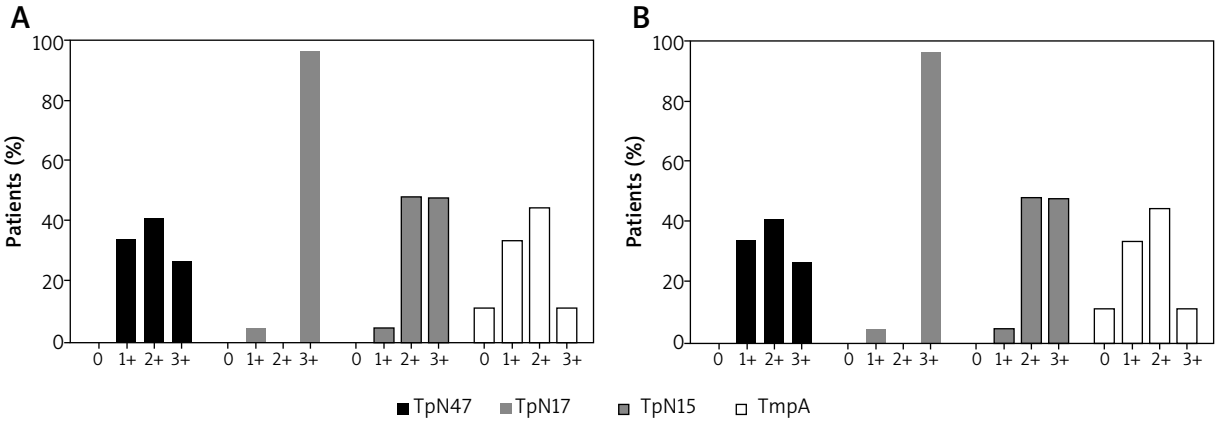

Figure 1. A – Percentages of patients with pre-treatment positive rates for specific anti-treponemal antibodies (TpN47, TpN17, TpN15, TmpA) stratified according to the reaction intensity (0, 1+, 2+, 3+). **B** – Percentages of patients with post-treatment positive rates for specific anti-treponemal antibodies (TpN47, TpN17, TpN15, TmpA) stratified according to the reaction intensity (0, 1+, 2+, 3+)

Table 2. Characteristics of pre-treatment specific treponemal antibody reactivity and ANAs positivity among individuals from the serofast state and serologically-cured groups

Parameter	Serofast state group (n = 10)	Serologically-cured group (n = 38)	*P*-value
Baseline TpN47, *n* (%):			0.0001
1+	7 (70)	2 (5.3)	
≥ 2+	3 (30)	36 (94.7)	
Baseline TpN17, *n* (%):			NS
1+	0 (0)	2 (5.3)	
≥ 2+	10 (100)	36 (94.7)	
Baseline TpN15, *n* (%):			NS
1+	0 (0)	2 (5.3)	
≥ 2+	10 (100)	36 (94.7)	
Baseline TmpA, *n* (%):			NS
1+	4 (40)	12 (31.6)	
≥ 2+	6 (60)	26 (68.4)	
Post-treatment ANAs positivity, *n* (%)	10 (100)	2 (5.3)	0.0001
ANA titer	1 : 640 (1 : 160–1 : 2560)	1 : 160	0.005

ANA – anti-nuclear antibodies, NS – not significant. The values are presented as medians (min.–max. range) if not otherwise stated.

Serum antibody reactivity to protein antigens of *T. pallidum* among the serofast state and serologically-cured groups

The percentage of patients with weak pre-treatment antibody reactivity to TpN47 (i.e. 1+) was significantly higher in the serofast state group when compared to those from the serologically-cured group ($p < 0.005$, Table 2). Weak (i.e. 1+) pre-treatment antibody reactivity to TpN47 was significantly associated with a higher risk of the serofast state (OR = 64; 95% CI: 5.01–817, $p < 0.05$). There were no differences in the analyzed groups in intensity of the reaction for TpN17, TpN15 and TmpA (Table 2). Similar results were found for post-treatment antibody reactivity to the profile of the analyzed treponemal antigens (data not shown).

ANA prevalence rates among serofast state and serologically-cured patients

All patients with the serofast state had positive ANA (i.e. titer ≥ 1 : 160). In comparison, the proportion of serologically-cured individuals with positive ANA was only 5.3% ($p < 0.05$). Interestingly, the mean ANA titer in the serofast state group was significantly higher when compared to those who had a proper serological response (1 : 640 and 1 : 160, respectively; $p < 0.05$). The most frequent ANA staining patterns were granular, followed by homogenous. In all cases, further confirmatory testing revealed that these anti-nuclear antibodies were non-specific.

Discussion

In the present study, the proportion of patients who had a serological non-response 6 months after treatment was 25%. This proportion decreased to 20.8% at 12 months after therapy. This value varies widely among other studies, and ranges from 9.4% to 44.4% [2]. Such a broad range can be explained in part by the variable proportion of different stages of syphilis among patients included in these studies and by an inconsistent definition of serological non-response. Noteworthy, we defined the serofast state to be lack of at least a four-fold decline in RPR titer after syphilis treatment. Some previous studies have found an association of serological outcomes with the stage of syphilis [7, 8]. In all of them, there was an increased likelihood of the serofast state among patients with ELS compared to those with the primary and secondary stage of the disease. The high proportion of serological non-responders observed in our study can be explained by the fact that 50% of patients included had ELS.

Some recent studies included lumbar punctures performed among individuals with serological non-response to syphilis therapy [9–11]. The proportion of patients who had reactive CSF suggestive of neurosyphilis among these studies varied from 5.7% to 34.6%. Such differences can be partially explained by the different criteria of neurosyphilis that were applied. Moreover, in some of these studies, patients were initially treated for syphilis with regimens that have not been proven to be effective in syphilis treatment, such as minocycline [9]. We performed CSF examination in all serofast state patients at 6 months after penicillin therapy and identified 2 out of 14 (14.3%) individuals who met the criteria for neurosyphilis. Our data suggest that neurosyphilis may be detected in patients despite the appropriate therapy for early-stage syphilis. Thus, clinicians should be aware of

these findings. Careful follow-up until proper serological response occurs is strongly recommended and the CSF examination in patients who remain serofast should be considered.

In previous studies, it was observed that sera samples of syphilitic patients showed reactivity against proteins TpN47, TpN17, TpN15 and TmpA, and that as the infection progressed, reactivity to all antigens increased [12–14]. Our results correspond well with those reported in previous studies. In the current study, patients with secondary syphilis and ELS had moderate or strong pre-treatment reactivity against the TpN17, TpN15 and TmpA antigens. It was previously shown that the TpN47 band is present in all phases of syphilis [14, 15]. Interestingly however, in early syphilis, the intensity of the reaction to TpN47 is more intense than in late syphilis, and can even become negative in later stages of the infection. Of note, we observed a significantly greater variation in staining intensity for TpN47 compared to the other antigens examined. The reactivity to TpN47 was assessed as weak, moderate or strong in 34%, 39% and 26% of all patients, respectively. Further analysis showed that the weak pre-treatment reactivity to TpN47 antigen was associated with a significantly higher risk of improper serological response at 12 months after completing the syphilis therapy. The mechanism underlying this observation remains unclear.

It has recently been suggested that a robust host pro-inflammatory immune response to the treponemal infection, occurring shortly after initiation of the treatment, is associated with a lower risk of maintaining the serofast state [16]. Thus, presumably, individuals with weak reactivity to TpN47 may have a down-regulated immune response, which results in an improper serological response. Further studies are needed in order to confirm this observation. We can only speculate that this down-regulation may be associated either with host genetic factors, such as single nucleotide polymorphisms affecting immune response to *T. pallidum,* or with an infection caused by less immunogenic treponemal strains.

Treponemal antibodies have been shown to persist in syphilis patients after therapy, indicating the presence of immune cells that continue to produce *T. pallidum* specific antibodies. Our results are consistent with those from the previous studies devoted to describing treponemal antigens in sera from treated patients [13, 14]. Twelve months after therapy we detected treponemal antibodies against all analyzed molecules (i.e. TpN47, TpN17, TpN15 and TmpA) even though the RPR titer had declined significantly or even reverted to be nonreactive. Interestingly, the band intensity remained almost unchanged. These and previous results suggest that methods for detecting treponemal antibodies may not be valuable for monitoring syphilis treatment.

In all patients with the serofast state, we found the presence of anti-nuclear antibodies (ANA) at a titer of 1 : 160 or above. In all these cases, confirmatory testing revealed that the ANA were not antigen specific. Interestingly, among individuals with a proper serological response there were only two cases with a positive ANA test. The ANA titer in all of these patients was low. Of note, none of individuals with ANA had complained of any symptoms characteristic of autoimmune disorders. The basis of this seropositivity in patients with a serofast state is puzzling. Nontreponemal antibodies are autoreactive since cardiolipin, the main antigenic component that binds to these antibodies, is present in mitochondrial membranes [6]. Thus, one of the explanations may be that this persistent presence of the nontreponemal antibodies in serofast patients leads to the destruction of host tissues and leads to production of ANAs. The question of why in some patients, this excessive production of nontreponemal antibodies occurs and whether persistence of nontreponemal antibodies after effective therapy represents failure of the immune system tolerance or lack of the pathogen clearance, remains unanswered. We are also uncertain as to whether ANAs are produced as the treponemal infection progresses, for example, in some genetically predisposed individuals, leading then to host tissues damage and to induction of nontreponemal antibodies production.

One of the important findings in our study was the detection of a high percentage (18.8%) of HIV co-infection in patients with syphilis who were unaware of their HIV status. This finding highlights the need for frequent HIV screening in individuals with syphilis.

All individuals in our study who were serologically non-responsive at 6 months after the initial therapy received an additional dose of benzathine penicillin. Of these patients, 83.3% still failed to have an appropriate serological response 6 months after retreatment (12 months after initial therapy). These findings suggest that persistent nontreponemal antibody titers may not be due to the insufficient therapy, but rather an alternative process like the immune response to the treponemal infection. It also implies that retreatment of serological non-responders does not lead to improved outcomes.

Our study has some limitations. Given the small sample size of this study and patients only with secondary syphilis and ELS included, we acknowledge that our data need to be interpreted with caution. Secondly, we showed that immune response to *T. pallidum* in serofast patients seems to be down-regulated with respect to reactivity to TpN47. However, the mechanism underlying this observation remains unclear. Further studies devoted to determining host factors that can lead to this down-regulation are needed. Thirdly, we assessed the presence of anti-nuclear antibodies after syphilis treatment in all included patients. Thus, we are unsure whether ANA appearance is a consequence of the spirochetal infection or they were present before the infection.

Conclusions

Our data support the hypothesis that the baseline antigen-specific immune response to the treponemal infection may be an important predictor of the treatment outcome. Some autoimmune reactions, not well characterized until now, may also be involved in the pathomechanism of the serofast state. Further studies on larger groups are needed in order to confirm our observations and evaluate the role of the immune system in these findings.

Conflict of interest

The authors declare no conflict of interest.

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